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Molecular characterization and hypoxia-induced upregulation of neuronal nitric oxide synthase in Atlantic croaker: Reversal by antioxidant and estrogen treatments

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ABSTRACT

Neuronal nitric oxide synthase (nNOS) catalyzes production of nitric oxide in vertebrate brains. Recent findings indicate that endothelial NOS and reactive oxygen species (ROS) are significantly increased during hypoxic stress and are modulated by antioxidants. However, the influence of antioxidants and steroids on nNOS upregulation by hypoxia is largely unknown. In this study, we characterized nNOS cDNA and examined the effects of hypoxia and antioxidant and steroid treatments on nNOS expression in Atlantic croaker hypothalamus. Hypoxia exposure (dissolved oxygen, DO: 1.7 mg/L for 2 and/or 4 weeks) caused significant increases in hypothalamic nNOS mRNA, protein and its neuronal expression. Hypothalamic nNOS expression and superoxide radical ($O_2^{\bullet -}$, an index of ROS) production were increased by pharmacological treatment of fish exposed to normoxic conditions with *N*-ethylmaleimide, an alkene drug which covalently modifies sulfhydryl groups and inhibits aromatase activity. In contrast, treatments with *N*ω-nitro-L-arginine methyl ester, a competitive NOS-inhibitor, or vitamin E, an antioxidant, prevented the upregulation of $O_2^{\bullet -}$ production and nNOS expression in hypoxia-exposed (DO: 1.7 mg/L for 4 weeks) fish. Moreover, treatment with 1,4,6-androstatrien-3,17-dione, an aromatase inhibitor, increased hypothalamic $O_2^{\bullet -}$ production and nNOS expression in normoxic control fish; whereas estradiol-17β treatment significantly reduced $O_2^{\bullet -}$ production and nNOS expression in hypoxia-exposed fish. Double-labeled immunohistochemical results showed that nNOS and aromatase proteins are co-expressed in the hypothalamus. Taken together, the results suggest that upregulation of nNOS and ROS in the croaker hypothalamus in response to hypoxia is influenced by antioxidant and overall estrogen status.

1. Introduction

Low levels of oxygen (dissolved oxygen, DO: <2 mg/L called hypoxia) and insufficient antioxidant levels in the vertebrate brain results in the initiation of a complex series of pathological changes which leads to decreased energy levels and disruption of neuroendocrine functions (Janero, 1991; Lahiri et al., 2006; Traber and Stevens, 2011). A major pathological pathway leading to neuronal dysfunction such as apoptosis and neurodegeneration involves overproduction of cellular reactive oxygen species (ROS) and nitric oxide (NO) (Beckman and Koppenol, 1996; Rosselli et al., 1998; Estévez and Jordán, 2002; Brown, 2010). NO acts as a reactive free radical and reacts directly with superoxide anion ($O_2^{\bullet -}$) to form peroxynitrite ($ONOO^-$) (Andrew and Mayer, 1999; Guittet et al., 1999). $ONOO^-$ and other related reactive nitrogen species (RNS) cause nitration of DNA, proteins and lipids, leading to increased oxidative damage and irreversible modification of neuronal constituents (Cazevieille et al., 1993; Tagami et al., 1998; Yamagata et al., 2010).

Nitric oxide synthases (NOSs) are multienzyme complexes which act on L-arginine, molecular oxygen and NADPH to produce L-citrulline, $NADP^+$ and NO. This process requires several catalytic domains, cofactors, and cations (see Supplementary Fig. 1A, Griffith and Stuehr, 1995; Förstermann and Sessa, 2012). In mammals, three distinct NOS isofoms have been characterized based on their structure, localization, regulation and catalytic functions (Alderton et al., 2001). These isoforms are designated as neuronal NOS (nNOS) (Bredt et al., 1991), inducible NOS (iNOS) which is induced by stress or inflammation (Mungrue et al., 2003), and endothelial NOS (eNOS) which is present in the brain vasculature (Marsden et al., 1992; Stanarius et al., 1997).

The structure, characteristics, and catalytic functions of NOS enzymes have been well studied in tetrapods (Alderton et al., 2001),

Abbreviations: nNOS, neuronal nitric oxide synthase; ROS, reactive oxygen species; RNS, reactive nitrogen species; NO, nitric oxide; NOx, nitrates and nitrites; Vit E, vitamin E; AOX, antioxidant; $O_2^{\bullet -}$, superoxide radical; $ONOO^-$, peroxynitrite; E2, estradiol-17β; ER, estrogen receptor; GRP30, G protein coupled receptor 30; ATD, 1,4,6-androstatrien-3,17-dione; CTK, cytokines; IL-1β, interleukin-1β; NEM, *N*-ethylmaleimide; SH, sulfhydryl; NAME, *N*ω-nitro-L-arginine methyl ester; RT-PCR, reverse-transcription polymerase chain reaction; RACE, rapid amplification of cDNA ends; GSP, gene specific primer; UTR, untranslated region; ORF, open reading frame; FMN, flavin mononucleotide; FAD, flavin adeninedinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; DO, dissolved oxygen.

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whereas comparable information on the NOS enzymes is lacking in teleost fishes. At present, only two NOS isoforms have been fully characterized in several teleost fishes: nNOS in killifish (Hyndman et al., 2006), red drum (Zhou et al., 2009) and catfish (Yao et al., 2014); and iNOS in common carp (Saeij et al., 2000), rainbow trout (Wang et al., 2001) and catfish (Yao et al., 2014). There is currently no sequence information on fish eNOS mRNAs. Nevertheless, immunoreactive expression of eNOS protein has been detected in the heart of zebrafish (Fritsche et al., 2000), caudal peduncle of tilapia (Cioni et al., 2002), gill of salmon (Ebbesson et al., 2005), head kidney of rainbow trout (McNeill and Perry, 2006), liver tissues of Atlantic croaker (Rahman and Thomas, 2012) and ovary of catfish (Singh and Lal, in press). However, eNOS has not been detected in fish brains suggesting that eNOS may be not involved in NO production in the teleost brain. On the other hand, although nNOS is traditionally considered a neuronal-specific isoform; it has also been detected in the gill, head kidney, intestine, spleen as well as other peripheral tissues of fishes (Hyndman et al., 2006; McNeill and Perry, 2006; Zhou et al., 2009), suggesting that nNOS is probably the only constitutively expressed NOS gene in teleost fishes. Evidence also suggests that nNOS is highly regulated at both the transcriptional and translational levels, and is one of the most important contributors to NO production during hypoxic stress (Mauceri et al., 2002; McNeill and Perry, 2006). Moreover, nNOS is also capable of producing $O_2^{\bullet -}$ and $ONOO^-$ under certain stress conditions (Andrew and Mayer, 1999). However, despite its important role in pathological responses to hypoxia and other stressors, the regulation and functions of nNOS are not yet well understood in teleost brains during hypoxia exposure.

Hypoxia interferes with physiological functions in vertebrates by inducing ROS and RNS through activation of NOS enzymes (Li and Jackson, 2002). Hypoxia increases nNOS activity, mRNA and protein levels, and neuronal expression in rat and porcine brains (Prabhakar et al., 1996; Yamamotoa et al., 2003; Ward et al., 2005; Mishra et al., 2006; McLaren et al., 2007; Tsui et al., 2011). Hypoxia also increases iNOS and eNOS expression in tetrapod tissues (Grilli et al., 2003; Ducsay and Myers, 2011). Studies in teleosts have shown that hypoxia increases nNOS mRNA and protein expressions in the posterior cardinal vein, and these increases in nNOS expression are accompanied by increases in plasma levels of nitrates and nitrites (NOx, a metabolite of NO) in rainbow trout (McNeill and Perry, 2006). We have recently demonstrated that hypoxia increases plasma NOx levels, eNOS protein expression and $O_2^{\bullet -}$ production in croaker liver (Rahman and Thomas, 2011, 2012; unpub. obs.). Hypoxia drastically increases cellular ROS and RNS production and decreases antioxidant activity (Koskenkorva-Frank et al., 2013). Antioxidants exert protective effects against oxidative stress and prevent the propagation of ROS and RNS (Chow, 1991; Chow et al., 1999; Traber and Stevens, 2011). Therefore, it is likely that during hypoxia exposure, insufficient antioxidant capacity in the tissues to scavenge the increased formation of ROS and RNS may be an important contributing factor to neuronal dysfunction. However, there is a lack of information on the role of antioxidants in nNOS regulation and endogenous ROS/RNS generation in vertebrate brains during hypoxia exposure.

Hypoxia disrupts neuroendocrine functions by interfering with neuroenzyme activities and altering neurotransmitter levels in the vertebrate brain (Shang et al., 2006; Kumar, 2011; Thomas et al., 2007; Thomas and Rahman, 2012). Recent *in vivo* studies in several teleost fishes have shown that hypoxia suppresses the enzymatic activity of aromatase in the brain and decreases plasma estradiol-17β (E2) levels (Shang et al., 2006; Thomas et al., 2007; Thomas and Rahman, 2012). Our recent laboratory studies have shown that the hypoxia-induced marked inhibition of hypothalamic aromatase activity and reduction in plasma E2 levels is associated with a decrease in the gonadal development in croaker (Thomas and Rahman, 2012). We have also shown that hypoxia drastically decreases tryptophan hydroxylase (TPH) activity and causes a decline in serotonin levels in croaker hypothalamus (Rahman and Thomas, 2009, 2014). On the other hand, administration of E2 increases TPH activity and serotonin levels in croaker hypothalamus under hypoxic conditions (Rahman and Thomas, 2014), which suggests that sufficient endogenous E2 levels are essential for maintaining serotonergic functions. *In vivo* studies in tetrapods have shown that E2 decreases nNOS neuronal expression in the hypothalamic paraventricular nuclei and the anterior pituitary in rat brains under normoxic conditions (Qian et al., 1999; Gingerich and Krukoff, 2005). Treatment with E2 also decreases nNOS protein expression of human neutrophils *in vitro* (García-Durán et al., 1999; Molero et al., 2002). Other studies demonstrated that E2 treatment does not alter nNOS mRNA and protein levels and neuronal expression in the magnocellular, supraoptic and paraventricular nuclei (Ceccatelli et al., 1996; Wang and Morris, 1999) in rat brains, suggesting that the effects of E2 on nNOS expression in the vertebrate brain appear to be regionally specific.

The aims of the present study were four-fold. Our first aim was to characterize Atlantic croaker nNOS cDNA and examine the profiles of nNOS mRNA, protein and neuronal expression in response to different periods of exposure to hypoxia (DO: 1.7 mg/L for 2 and/or 4 weeks) in croaker hypothalamus. Atlantic croaker is a relatively hypoxia-tolerant marine teleost that inhabits estuarine and coastal regions along the US Atlantic and Gulf of Mexico coasts as well as the northern Gulf of Mexico that are often hypoxic during summer (Thomas et al., 2007; Thomas and Rahman, 2012). Severe and widespread impairment of reproductive and endocrine functions have been observed in croaker collected from hypoxic region in the northern Gulf of Mexico (Thomas et al., 2007; Thomas and Rahman, 2012). A second aim was to determine the role of vitamin-E, a potent antioxidant which regulates neuronal function(s) and maintains cellular integrity (Muller, 2010), on $O_2^{\bullet -}$ production and nNOS expression in croaker hypothalamus under both hypoxic and normoxic conditions. An alkylating drug, *N*-ethylmaleimide (NEM), which covalently modifies sulfhydryl groups to produce NO, and a NOS-inhibitor, *N*ω-nitro-L-arginine methyl ester (NAME), were used in normoxic and hypoxic conditions in order to determine whether $O_2^{\bullet -}$ is generated through a NOS-dependent pathway in response to hypoxia. The third aim was to compare the effects of chronic treatment with the aromatase-inhibitor, 1,4,6-androstatrien-3,17-dione (ATD) to those induced by hypoxia exposure on $O_2^{\bullet -}$ production and nNOS mRNA and protein expression in croaker hypothalamus. We also investigated whether chronic E2 treatment reverses these changes in hypoxia-exposed fish to levels observed under normoxic conditions. Finally, co-localization of nNOS and aromatase proteins in croaker hypothalamus were investigated to provide a potential neuroanatomical basis for any close interactions observed between these two neuroenzymes.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), TRI reagent, *N*-ethylmaleimide (NEM), *N*ω-nitro-L-arginine methyl ester (NAME), 1,4,6-androstatrien-3,17-dione (ATD), quinaldine, EGTA and EDTA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibody against nNOS and rabbit anti-actin IgG horseradish peroxidase (HRP)-linked antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Novus Biologicals (Littleton, CO, USA), respectively. Rabbit polyclonal anti-aromatase antibody was a generous gift from Dr. Andrew H. Bass, Cornell University, Ithaca, NY, USA, and the specificity of antibody has been demonstrated in teleost brain previously (Forlano et al., 2006). Oligonucleotides were synthesized by Eurofins MWG Operon (Huntsville, AL, USA). Materials for molecular biology were purchased from Agilent Technologies (La Jolla, CA, USA), Promega (Madison, WI, USA), and Invitrogen (Carlsbad, CA, USA). All other chemicals were obtained from Sigma-Aldich and Fisher Scientific (Pittsburgh, PA, USA) unless noted otherwise.

2.2. Experimental fish

Adult young (year 1) Atlantic croaker, *Micropogonias undulatus* (length: 10–11 cm, body weight, BW: 12–18 g), were purchased from local fisherman collected by shrimp trawl in the Aransas Bay, Texas, USA. Fish were transported to nearby fish holding facilities at the University of Texas Marine Science Institute and treated with Paracide-F (Argent Chemical, Redmond, WA, USA) at 170 ppm in seawater for 1 h to minimize parasite infections. Fish were transferred to large indoor tanks (4727 l) equipped with a recirculating seawater system (salinity 30–32 ppt) and maintained under control photoperiod (11L:13D) and temperature (22 ± 1 °C) conditions. Fish in each tank were fed chopped shrimp daily (3% BW/day) and acclimated in fully aerated recirculating seawater to laboratory conditions for at least 3 months prior to experimentation during the period of gonadal recrudescence.

2.3. Ethics statement

All the following experimental procedures were approved by the University of Texas at Austin Animal Care and Use Committee (IACUC, protocol# 09022701).

2.4. Experiment 1: effects of hypoxia exposure (2 and 4 weeks) of nNOS regulation

A detailed account of the hypoxia-exposure methods used in this study has been described previously (Rahman and Thomas, 2009). Briefly, adult fish were stocked into each of eight tanks (30 mixed-sex fish per tank) with a recirculating seawater system and fed chopped shrimp daily (3% BW/day) during the experimental period. Fish were exposed to normoxic (dissolved oxygen, DO: 6.5 mg/L) or hypoxic conditions under controlled environmental conditions that mimicked the seasonal changes in photoperiod (11L:13D) and temperature (22 ± 1 °C). Hypoxic conditions in the tanks were maintained by reducing the aeration gradually through the air flow system and finally adjusted until the DO level reached 1.7 mg/L which was achieved within 2 days. At the end of experiments, fish were sacrificed by exposing them to quinaldine (20 mg/L; Sigma-Aldrich) for five minutes. Brain tissues were quickly excised, frozen in liquid nitrogen and stored at −80 °C for later excision of hypothalamic tissue for RNA extraction and protein determination. For immunohistochemical detection, brain samples were fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight at 4 °C.

2.5. Experiment 2: effects of hypoxia and pharmacological agents on nNOS regulation

Adult fish were stocked into each of six tanks (30 mixed-sex fish/tank) and exposed to normoxia (DO: 6.5 mg/L) or hypoxia (DO: 1.7 mg/L) under the controlled laboratory environmental and husbandry conditions as described in experiment 1. Fish were anesthetized with quinaldine (20 mg/L) for 1–2 min and given an intraperitoneal injection (i.p.) either with vehicle, NEM, NAME or vitamin E every 4 days for 4 weeks (6 injections of 1 μg/g BW). Fish exposed to normoxia were treated with the alkylating agent, NEM, to determine whether it mimics the effects of hypoxia on superoxide radical ($O_2^{\bullet -}$, an index of ROS) or nNOS expression. Fish exposed to hypoxia were treated with the NOS inhibitor, NAME, to determine if it impaired $O_2^{\bullet -}$ or nNOS responses to hypoxia. Fish were sacrificed 4 days after the last injection. Brain tissues were quickly excised, stored at −80 °C, and processed as described previously at the end of experiment for measurement of RNA and protein expression in hypothalamic tissue. For measurement of $O_2^{\bullet -}$, hypothalamic tissues were excised from fresh brain samples.

2.6. Experiment 3: effects of hypoxia and E2 or ATD treatments on nNOS regulation

Thirty-mixed sex adult fish (sex could not be determined prior to gonadal recrudescence) were stocked into each of six tanks and exposed to normoxic (DO: 6.5 mg/L) and hypoxic (DO: 1.7 mg/L) conditions (same as experiment 2). Fish were anesthetized with quinaldine and injected i.p. either with vehicle, E2 or ATD every 4 days for 4 weeks (6 injections of 1 μg E2 or ATD/g BW). Fish were sampled 4 days after the last injection. Brain tissues were quickly excised, stored at −80 °C, and processed as described previously at the end of experiment for measurement of RNA and protein expression in hypothalamic tissue, and $O_2^{\bullet -}$ production.

2.7. Cloning and sequencing of nNOS cDNA

Molecular protocols used for RNA extraction, reverse transcriptase-polymerase chain reaction (RT-PCR) amplification, DNA purification, and cloning were similar to those of Rahman and Thomas (2012). Briefly, total RNA was extracted from brain tissues using 1 ml TRI reagent (Sigma-Aldrich) and treated with DNase (Promega) according to the manufacturer's protocol to eliminate genomic DNA. First-strand cDNA synthesis was carried out using Superscript III reverse transcriptase (Invitrogen). A partial cDNA of nNOS was obtained by PCR amplification. Primers for amplifying the cDNA fragment were designed to span highly conserved regions of the known sequences of nNOS in teleost fishes (Table 1). The PCR product was separated by 1% agarose gel, purified, ligated into pGEM-T easy vector (Promega), transformed into competent cells (Promega) and sequenced (Institute for Cellular and Molecular Biology Core Research DNA Sequencing Facility, University of Texas at Austin). The full-length sequence of croaker nNOS was obtained with 5′- and 3′-RACE amplification kits (Invitrogen) using gene specific primers (Table 1).

2.8. Sequence alignment, structural modeling and phylogenetic analysis

The deduced amino acid sequence of croaker nNOS cDNA was aligned with known vertebrate nNOSs published in GenBank. Alignments of nNOS cDNAs were conducted using ClustalW (1.83) (http://www.ebi.ac.uk/Tools/clustalw/index.html).

Structural modeling (Henrich et al., 2009; Wass et al., 2010) and sequence analysis were conducted to examine the molecular basis of cross-activity between oxygenase and reductase domains binding sites in the croaker nNOS protein. The predicted three-dimensional (3D) structure models of the oxygenase and reductase domains of croaker nNOS protein were designed by Iterative Threading ASSEmbly Refinement (I-TASSER, a bioinformatics method for predicting 3D structure model of protein molecules; http://zhanglab.ccmb.med.umich.edu/I-TASSER/) method according to Zhang (2008) and Roy et al. (2011).

Table 1
Primers used for cloning of croaker nNOS cDNA.

Name of primer	Sequence for nNOS primer
Primers for partial sequence	
PSP-F	5′-CCCTTCAGTGGCTGGTACAT-3′
PSP-R	5′-CTCTTCAAACAGCGTGTCCA-3
Primer for 5′ RACE	
GSP 5′-1	5′-GTCGCAGAAGTCTCTCACACCAATCT-3′
GSP 5′-2	5′-ATCTCCACAAGTGCCTGGTCTTTC-3
GSP 5′-3	5′-GGAAGTCATGTTTGCCATCTGTCC-3′
GSP 5′-4	5′-TTAGCAGGGTCACCCATGATCTGT-3′
Primers for 3′ RACE	
GSP 3′-1	5-CACCCAACATCCAACACAGAAGAC-3′
GSP 3′-2	5′-CCAACGTCAGGTTCTCAGTGTTTG-3′

Abbreviations: PSP, partial sequence primer; GSP, gene-specific primer; RACE, rapid amplification of cDNA ends.

These models were subjected to minimization of 3000 Steepest-Descent steps using Discovery Studio 3.5 software (http://accelrys.com/products/discovery-studio/requirements/technical-requirements-350.html).

A phylogenetic tree was constructed of nNOS, iNOS and eNOS cDNAs using the Neighbor-Joining method according to Tamura et al. (2007). The consensus tree was developed using the MEGA6 software package (http://megasoftware.net). Bootstrap analysis (1000 replicates) was performed to assess the degree of strength for branches on the tree. Only sequences that included a full coding cDNA sequence were used in the analysis.

2.9. Tissue distribution of nNOS mRNA by RT-PCR

Total RNA was extracted from croaker brain, eye, gill, heart, kidney, intestine, liver, testis, and ovary using TRI-reagent followed by DNase treatment to remove any genomic DNA contamination. RT was performed using Superscript III reverse transcriptase (Invitrogen) at 50 °C for 1 h. Thirty five cycles of PCR reactions were performed using Green *Taq* PCR MasterMix (Promega) in a Mastercycler (Eppendorf, Hamburg, Germany) and the following primers: sense, 5′-CCCTTCAGTGGCTGGTA CAT-3′, and antisense, 5′-CTCTTCAAACAGCGTGTCCA-3′. The PCR cycling profiles were 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 10 min. To visualize the PCR products, 1% agarose gel electrophoresis was performed following standard procedures. Lack of genomic DNA contamination was also confirmed by PCR reactions in the absence of RT.

2.10. Western blot analysis

Proteins were extracted from hypothalamic tissues with a TRI reagent-guanidine technique, a method for the extraction of high-quality RNA and protein from a single sample, according to Rodrigo et al. (2002). Protein samples (2 μg) were solubilized by boiling in sodium dodecyl sulfate (SDS, Sigma-Aldrich) loading buffer (0.5 M Tris–HCl, 0.5% bromophenol blue, 10% glycerol) and cooled on ice for 5 min. Proteins were then electrophoresed on a 10% SDS-PAGE gel and transferred onto a immuno-blot polyvinyl difluoride membrane (PVDF membrane, Bio-Rad) for 1 h at 4 °C. After transfer, membranes were blocked with 5% nonfat milk in Tris-buffer saline (TBS-T: 50 mM Tris, 100 mM NaCl, 0.1% Tween 20, pH 7.4) for 2 h, washed with TBS-T buffer and probed with primary nNOS antibody (dilution: 1:1000) with 5% nonfat milk overnight at 4 °C. The nNOS antibody has been validated in teleost brain previously (McNeill and Perry, 2006). Membranes were washed with TBS-T buffer and incubated with goat anti-rabbit IgG HRP-linked secondary antibody (1: 4000; Southern Biotech, Birmingham, AL, USA) for 2 h. Membranes were then rinsed with TBS-T buffer, visualized with an enhanced chemiluminescence substrate (Pierce, Rockford, IL, USA), and exposed to X-ray film (Amersham Biosciences, Buckinghamshire, UK) in the dark. The intensity of the nNOS and actin protein bands was estimated using ImageJ software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). Actin protein (~45 kDa) was used as an internal control to normalize sample loading on the gels.

2.11. Quantitative real-time PCR (qRT-PCR) analysis

nNOS mRNA levels were determined by qRT-PCR on total RNA using a one step Brilliant SYBR Green qRT-PCR master mix (Agilent Technologies, La Jolla, CA, USA) as described previously (Rahman and Thomas, 2012, 2013). Briefly, gene-specific primers for croaker nNOS (sense: 5′-CTGGAGACTGGGTGTGGATT-3′ and antisense: 5′-TCTTGAATCCGATC GCTCTT-3′; GenBank accession number KM067455) were used for amplification of croaker nNOS mRNA expression. The RNA samples were assayed in a 25-μl reaction mixture containing 12.5 μl 2× SYBR-qRT-PCR master mix, 50 nM of gene specific primers, 0.063 μl StrataScript RT/RNase block enzyme mixture, and 250 ng of total RNA. The qRT-PCR cycling profiles were 50 °C for 30 min, 95 °C for 10 min, and 40 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 30 s. Melting curve analysis was also performed at 1 cycle of 95 °C for 1 min, 50 °C for 30 s, and 95 °C for 30 s. nNOS transcript level was normalized on the basis of the quantification of croaker 18S rRNA (primers: sense 5′-AGAAACGGCTACCACATCCA-3′ and antisense 5′-TCCCG AGATCCAACT ACGAG-3′; AY866435). RT-negative qRT-PCR reactions were also conducted to ensure the qRT-PCR results were not the product of genomic DNA contamination. The relative nNOS mRNA expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.12. Northern blot analysis

Details of the Northern blot analysis methods were used in this study have been described previously (Rahman and Thomas, 2009). Briefly, total RNA (20 μg) samples were electrophoresed on 1% (w/v) agarose/formaldehyde gels in MOPS buffer (20 mM MOPS, 2 mM sodium acetate and 1 mM EDTA, pH 7.0) and blotted onto nylon membranes for 2 h. The membranes were briefly rinsed in running buffer to remove agarose gel and treated by baking at 80 °C for 15 min in a hybridization incubator (Fisher Scientific). The membranes were then prehybridized in prehybridization buffer (Ambion, Austin, TX, USA) at 68 °C for 30 min. Digoxigenin (DIG)-labeled RNA probes were synthesized using a DIG RNA labeling mix (Roche Diagnostics, Penzberg, Germany) and diluted in prehybridization buffer. Hybridizations were performed overnight at 68 °C with 100 ng of a labeled RNA probe in hybridization buffer. Membranes were then washed with low and high stringency washing buffer at 68 °C for 15 min and incubated with a DIG-labeled antibody (1:20,000) for 30 min. Membranes were exposed to X-ray film (Amersham Biosciences, Buckinghamshire, UK) to detect the specific signal using the DIG luminescent detection kit (Roche Diagnostics).

2.13. Single- and double-immunofluorescence staining of nNOS and aromatase proteins

Single-immunofluorescence staining methods were performed according to Rahman and Thomas (2009, 2013). Briefly, whole brains were fixed in 4% paraformaldehyde solution overnight at 4 °C, dehydrated in ethanol solutions, embedded in paraffin, sectioned at 10 μm, and mounted in poly-L-lysine coated slides. Sections were deparaffinized in xylene, dehydrated in ethanol solutions, and rinsed with PBS. Slides were then incubated in blocking buffer (PBS containing 0.3% Triton X-100, 1% BSA, and 5% normal rabbit serum) for 1 h at room temperature prior to incubation with nNOS antibody (1:100 dilution in blocking buffer) overnight at 4 °C. The specificity of the nNOS antibody has been validated in croaker brain previously (Rahman and Thomas, 2014). The immunofluorescent signals of nNOS neurons in the croaker hypothalamus were amplified with a tyramide signal amplification (TSA) solution (Molecular Probes, Eugene, OR) using a signal-labeling technique according to the manufacturer's protocol. The image was captured by Cool-SNAP camera (Photometrics, Tucson, AZ, USA). The immunofluorescence signal intensity of each neuron was quantified after subtracting the background signal using ImageJ software according to Collins (2007).

A double-immunofluorescence method was used to detect nNOS and aromatase in the same neurons. This method, which employs two primary antibodies raised in the same species, has been described by Shindler and Roth (1996) and Kroeber et al. (1998). Briefly, rabbit polyclonal anti-nNOS antibody was purified by Melon Gel IgG purification column (Thermo Fisher Scientific, Rockford, IL, USA) and directly labeled with DyLight-488 antibody (Thermo Scientific) according to the manufacturer's protocol. Sections were then incubated with unlabelled rabbit aromatase primary antibody (1:100) overnight at 4 °C, rinsed with PBS and incubated with fluorescent Rhodamine Red-X conjugated goat anti-rabbit secondary antibody (1:500; Jackson Immunoresearch, West Grove, PA, USA) for 1 h in the dark. Sections were rinsed with PBS, and incubated with 5% normal rabbit serum in PBS-T (PBS with

0.3% Triton X-100) to occupy any free binding site for rabbit antibody. Sections were then blocked with blocking solution (3% normal goat serum and 0.3% Triton X-100 in PBS) for 1 h, rinsed with PBS, and incubated with anti-nNOS-DyLight 488 labeled-antibody (1:500) at 4 °C overnight in the dark. Sections were then rinsed three times in PBS and mounted in Fluoromount-G solution (SouthernBiotech). The presence of the double-labeled immunofluorescence signal was visualized using a confocal microscope (Nikon Eclipse C2, Nikon, Japan).

2.14. Endogenous $O_2^{\bullet -}$ production

Endogenous $O_2^{\bullet -}$ production was determined as an index of ROS production in croaker hypothalamic tissues during reduction of ferricytochrome *c* according to Heim et al. (1991) and Drossos et al. (1995) with minor modifications. Briefly, fresh hypothalamic tissue samples were cut into thin slices and transferred in disposable glass culture tubes containing 3 ml of ice-cold Krebs buffer (2.5 mM $CaCl_2$, 118.2 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 11.2 mM glucose) in an ice bath. Cytochrome *c* (15 μmol) was then added to the sample in the culture tube and incubated at 37 °C in a water bath for 15 min. The reaction was stopped by placing the culture tubes in an ice bath. The samples were then centrifuged at 3500 *g* at 4 °C for 10 min and the absorbance of the supernatant was read at 550 nm in a spectrophotometer (DU-640; Beckman Instruments, Corona, CA, USA) after the addition of 3 mM NEM to inhibit further reduction of cytochrome *c*. Endogenous $O_2^{\bullet -}$ production was calculated from the conversion of ferricytochrome *c* to ferrocytochrome *c*, $E_{550} = 21$ nM/cm, and the results of $O_2^{\bullet -}$ production were expressed as nmol/min/g tissue.

2.15. Statistical analysis

All of the experimental results were analyzed by Student's *t*-test for unpaired comparisons and one-way analysis of variance (ANOVA) combined with Fisher's protected least-significant difference (Fisher's PLSD) test for multiple comparisons. Differences were considered at $P < 0.05$ significant level use by GraphPad Prism (GraphPad, San Diego, CA, USA) and StatView (SAS Institute Inc., Cary, NC, USA) software packages.

3. Results

3.1. Molecular cloning, characterization, structural and phylogenetic analyses of croaker nNOS

A distinct cDNA fragment (969 bp) of croaker nNOS was identified by partial cloning. The complete cDNA sequence of croaker nNOS was obtained by 3′- and 5′-rapid amplification of cDNA ends (RACE). The nucleic acid sequences of the RACE products were identical in the overlapping portions to the partial sequences. The full-length croaker nNOS cDNA consists of 4731 bp nucleotides which contains a 3′-untranslated region (3′-UTR, 108 bp), a 5′-UTR region (368 bp), and an open reading frame (4281 bp) encoding a polypeptide of 1427 amino acid residues (Fig. 1) with a predicted molecular weight of the protein at ~160 kDa. The nucleotide and amino acid sequences of croaker nNOS were deposited in GenBank (accession number KM067455) and were compared with those of other vertebrates (Fig. 1; Supplementary Fig. 1B).

The croaker nNOS sequence has two major domains, oxygenase (Fig. 1A) and reductase (Fig. 1C), are shown in Fig. 1 as superscript lines above the amino acid residues. Both domains show high sequence identities with other vertebrate nNOSs (oxygenase domain: 84–97% identity with other teleost nNOSs and 71–73% with those of tetrapods, Fig. 1B; reductase domain: 89–96% with teleosts and 73–80% with tetrapods, Fig. 1D). The N-terminal oxygenase domain contains postsynaptic density-95/discs large/ZO-1 (PDZ), L-arginine (ARG), HAEM and tetrahydrobiopterin (BH_4) binding sites. These are linked by a calmodulin (CaM)-recognition site to a C-terminal reductase domain containing flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH) binding sites. Like those of other teleosts, the croaker nNOS protein shows extensive sequence homologies in the oxygenase (96–100% for PDZ and 86–94% L-arginine/BH_4/HEME binding sites) and reductase (94–100% for FMNs and 85–98% FAD/NADPH binding sites) domains (Supplementary Fig. 1B).

The predicted 3D structure models of the oxygenase and reductase domains of croaker nNOS protein using I-TASSER are shown in Supplementary Fig. 2A and 2B, respectively. Confidence score for estimating the quality of predicted models are shown in Supplementary Fig. 2C.

The deduced amino acid sequence of croaker nNOS shows high sequence identity with that of red drum (96.7%), killifish (91%) zebrafish (85%), tilapia (93.8%) fugu (90%) medaka (90.5%), whereas its sequence identity is relatively low to Xenopus, rat and human at ~75% (Fig. 2A). A phylogenetic tree of the deduced amino acid sequences of nNOS, endothelial NOS (eNOS) and inducible NOS (iNOS) shows that the croaker nNOS amino acid sequence aligns with teleost nNOSs and is more closely related to the tetrapod nNOSs than to the corresponding eNOS and iNOS sequences (Fig. 2B).

3.2. Tissue specific expression of nNOS mRNA and protein

RT-PCR showed high expression of nNOS mRNA in the croaker brain followed by the kidney, eye, heart and gill. The nNOS mRNA was weakly expressed in the intestine, ovary, testis and liver, and was not detected in the muscle (Fig. 3A). No amplification of nNOS and 18S occurred in PCR reactions using RT-negative reactions (Fig. 3A), which indicates that genomic DNA did not serve as template in nNOS and 18S PCR reactions.

Western blot analysis using the nNOS polyclonal antibody showed immunoreactive bands of the predicted size ~160 kDa in croaker tissue extracts (Fig. 3B). The nNOS protein was highly expressed in the croaker brain (Fig. 3B). A weak band was apparent in the kidney and eye, and a faint band was also visible in the heart. No immunoreactive bands were detected in other tissue extracts. The specificity of the immunoreactive staining reaction with the nNOS antibody in the croaker brain was confirmed by blocking with the peptide antigen (Fig. 3C). Equal loading of the different tissue protein extracts was confirmed with the actin (predicted size ~45 kDa) as loading control (Fig. 3B).

3.3. nNOS mRNA and protein expression in discrete brain areas

Quantitative real-time PCR (qRT-PCR) results revealed that nNOS mRNA was more highly expressed in the preoptic-anterior hypothalamus (POAH), midbrain tegmentum (MT) and telencephalon (TEL) compared with other brain areas (Fig. 4A). Western blot results showed that the nNOS protein was highly expressed in the POAH and MT followed by the TEL and medulla oblongata. The nNOS protein was weakly expressed in the olfactory bulb and cerebellum plus optic tectum. No immunoreactive bands were detected in the pituitary (Fig. 4B-a). The specificity of the immunohistochemical (IHC) staining reaction with the nNOS antibody in the croaker hypothalamus was confirmed by blocking with specific peptide antigen (Fig. 4C-b). Northern blot analysis of the croaker nNOS transcript in the hypothalamus produced a single hybridization band of ~7.0 kb (Fig. 4D).

3.4. Effects of hypoxia on nNOS mRNA, protein and neuronal expression

qRT-PCR results showed that nNOS mRNA levels were significantly increased ~2-fold after short- (2 weeks) and long-term (4 weeks) hypoxia exposure (DO: 1.7 mg/L) compared to normoxic controls (DO: 6.5 mg/L) (Fig. 5A). The 18S threshold cycle (C*t*) values in croaker hypothalamus were the same in normoxic controls and hypoxia exposure groups (Supplementary Fig. 3). Strong expression of nNOS immunoreactivity was detected in hypothalamic neurons of fish exposed for 2- and 4-week to hypoxia, ~43% greater than that in normoxic controls (Fig. 5B, C).

A strong linear relationship between nNOS protein concentrations and immunoreactive band intensity on Western blots was confirmed with serial dilutions of nNOS and actin proteins (Fig. 6A, B) with correlation coefficients of 0.98 and 0.95, respectively (Fig. 6C, D). The intensities of the nNOS protein bands on Western blots were significantly ($P < 0.05$) increased after 2- and 4-week hypoxia exposure compared with normoxic controls following normalization to actin protein (Fig. 6E, F).

3.5. Interactive effects of hypoxia and NEM, NAME, or Vit E on ROS and nNOS regulation

To investigate the roles of nNOS, and antioxidant status on ROS generation and nNOS expression in response to hypoxia, we measured superoxide radical ($O_2^{\bullet -}$, a ROS) production in croaker hypothalamus with or without *N*-ethylmaleimide (NEM, an alkylating drug), *Nω*-

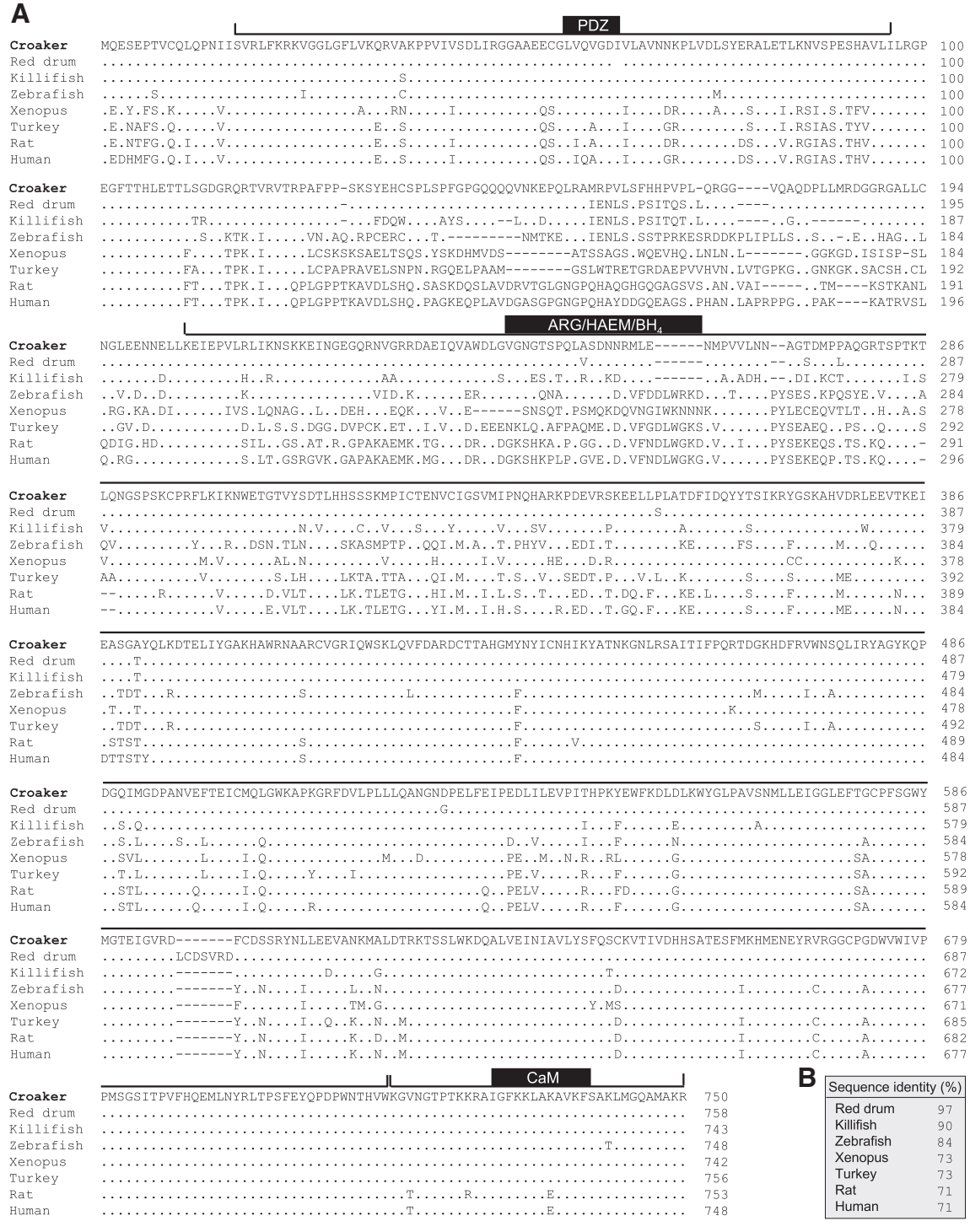

Fig. 1. Alignment of the full-length deduced amino acid sequence of oxygenase and reductase domains of croaker nNOS with the nNOS-related protein of other vertebrates. Dots indicate residues that are identical to croaker nNOS. Dashes indicate gaps introduced to facilitate alignment. The predicted binding sites of oxygenase (A) and reductase (C) domains in croaker nNOS protein with those of nNOSs from other vertebrates are shown overlined. These binding sites include: PSD-95/discs large/ZO-1 (PDZ), L-arginine (ARG), HAEM, tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmodulin (CaM), connecting (CoN) domains. (B, D) Percent identities of the deduced amino acid sequence of oxygenase and reductase domains of croaker nNOS protein with those of nNOSs from other vertebrates. GenBank accession numbers for the sequences of nNOSs used are as follows: Atlantic croaker (KM067455), red drum (ACU98970), killifish (AY533030), zebrafish (AY211528), *Xenopus* (AF053935), rat (P29476), turkey (XP_003211175), and human (NM_000620).

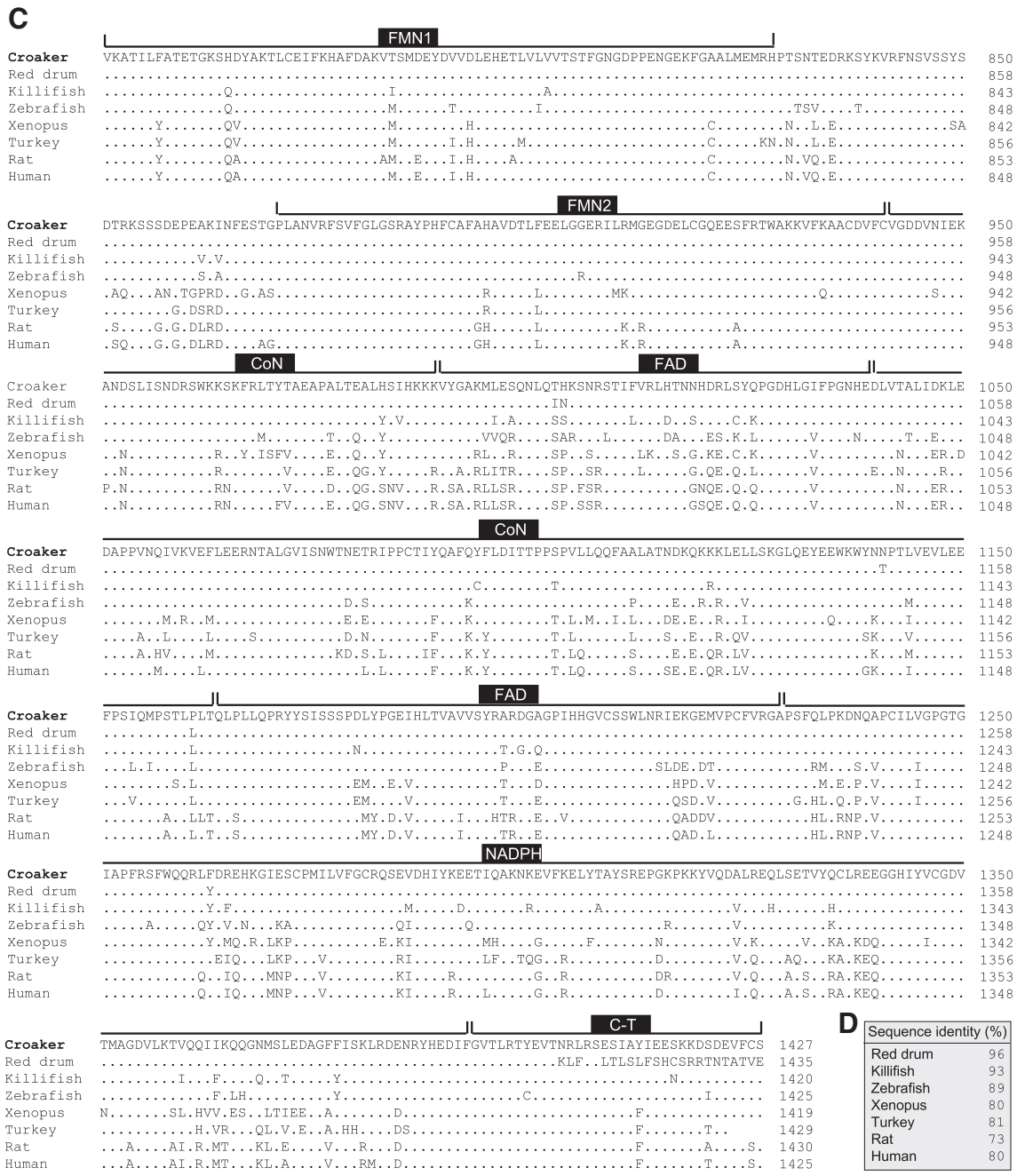

Fig. 1 (*continued*).

nitro-L-arginine methyl ester (NAME, a NOS inhibitor), or vitamin E, an antioxidant (AOX), treatment after 4 weeks of hypoxia (DO: 1.7 mg/L) exposure. There was a dramatic increase in hypothalamic $O_2^{\bullet -}$ production (~3-fold) in hypoxia-exposed fish compared with normoxic saline-injected controls (Fig. 7A). $O_2^{\bullet -}$ production was also significantly increased in normoxic fish by treatment with NEM similar to that observed in the hypoxia-exposed fish, whereas $O_2^{\bullet -}$ production was dramatically decreased in hypoxia-exposed fish by NAME or AOX treatment similar to that observed in the normoxic saline-injected control groups (Fig. 7A). In order to determine whether the changes on $O_2^{\bullet -}$ production were related to alterations in nNOS expression, we also investigated the effects of hypoxia and NEM, NAME, or AOX treatment on nNOS mRNA and protein expression. nNOS mRNA levels were markedly increased by treatment with NEM in normoxic fish (Fig. 7B). Injection with NEM did not significantly increase nNOS protein expression and relative protein levels in normoxic fish (Fig. 7C,D). nNOS mRNA and protein levels were also increased ~2.7-fold in the hypoxia-exposed saline-injected fish compared with normoxic saline controls, and these increases were completely blocked by the NAME and AOX treatments (Fig. 7B-D).

3.6. Interactive effects of hypoxia and ATD or E2 on ROS and nNOS regulation

To investigate the possible role of estrogen status on ROS generation and nNOS expression in response to hypoxia, we measured $O_2^{\bullet -}$ production in croaker hypothalamus with or without the aromatase inhibitor ATD, or estradiol-17β (E2) treatments. It should be noted that the treatment paradigm of E2 (1 μg E2/g BW) used in the present study results in plasma steroid hormone concentrations of 1–2 ng/ml (Khan and

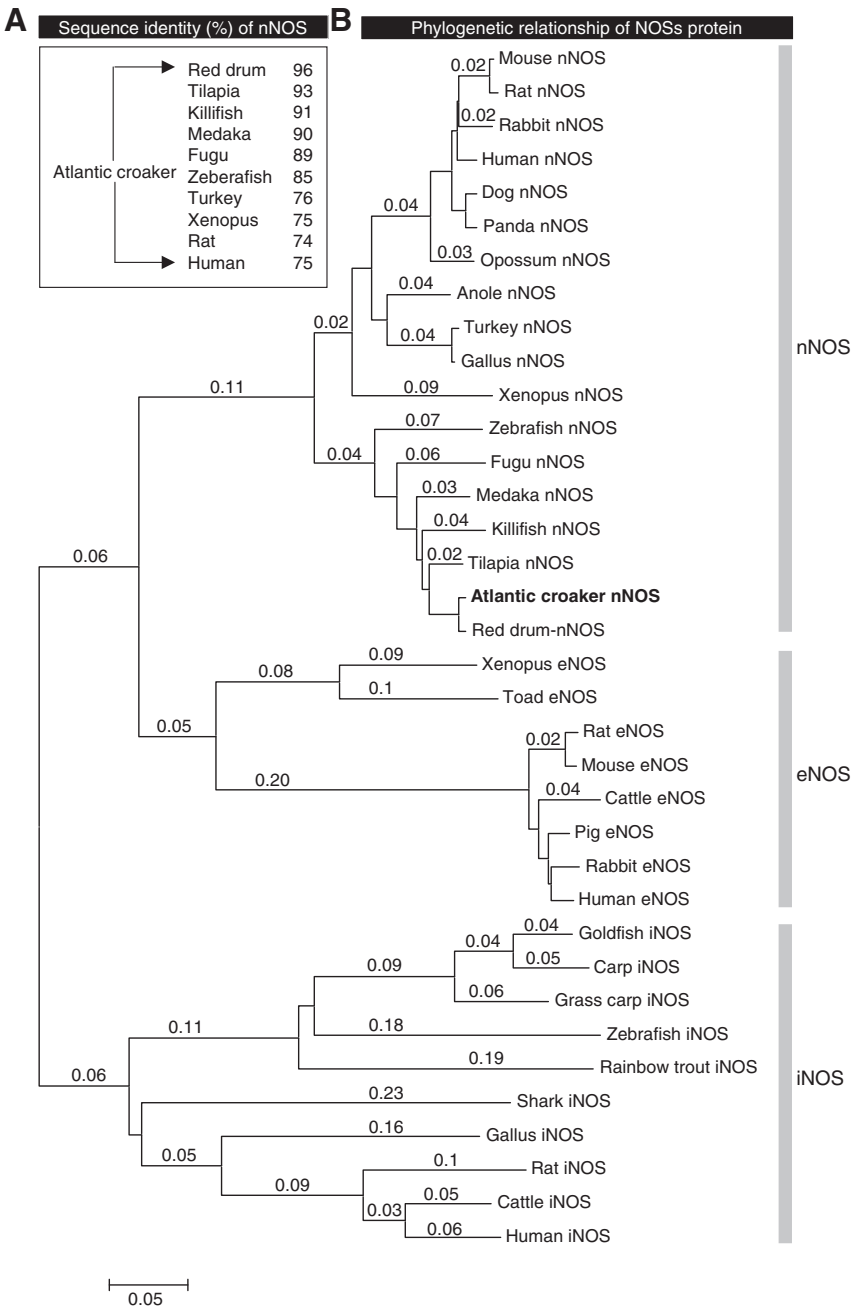

Fig. 2. Percent identities of the deduced amino acid sequence of croaker nNOS protein with those of nNOSs from other vertebrates (A). (B) Molecular phylogeny of nNOS, endothelial NOS (eNOS) and inducible NOS (iNOS) proteins. Scale bar indicates the number of changes inferred as having occurred along each branch. GenBank accession numbers for nNOS: tilapia (XP_003454198), fugu (AF380137), medaka (NP_001098325), mouse (D14552), rat (P29476), rabbit (NP_001075854), dog (NP_001182074.1), panda (XP_002920786.1), opossum (XP_001362705), anole (XP_003222831), gallus (XM425296), additional GenBank accession number of nNOS in Fig. 1; eNOS: mouse (AAC52766), rabbit (AAO47084.1), toad (ADK62525), rat (NP_068610), cattle (AAA30669), pig (AAR27960) and human (BAA05652.1); and iNOS: cattle (NP_001070267.1), human (BAA05531.1), rat (NP_036743.3), rainbow trout (AJ295231), zebrafish (NP_001098407.1), grass carp (ADT78701), goldfish (AY904362), carp (CAB60197.1), shark (AAX85385) and gallus (Q90703). Note: No teleost eNOS cDNA sequences are available in GenBank.

Thomas, 1993) which is at the high end of the physiological range for croaker (Thomas et al., 2007). No sex differences were observed in nNOS mRNA and protein levels in response to any of these treatments (see Supplementary Fig. 4). Therefore, the results of both sexes were combined. Exposure to hypoxia (DO: 1.7 mg/L for 4 weeks) and treatment with ATD (1 μg ATD/g BW/4 days for 4 weeks) as well as treatment of normoxic fish with ATD caused significant increases in hypothalamic $O_2^{\bullet -}$ production compared to normoxic saline-injected controls (Fig. 8A). Injection with E2 (1 μg E2/g BW/4 days for 4 weeks) had no effect on $O_2^{\bullet -}$ production in normoxic fish, but the hypoxia-induced increase in $O_2^{\bullet -}$ production was completely blocked by E2 treatment (Fig. 8A). nNOS mRNA levels were significantly increased in normoxic fish by treatment with ATD compared with normoxic saline-injected controls (Fig. 8B). Injection with ATD did not significantly affect nNOS protein levels in normoxic fish, but the hypoxia-induced increase in nNOS protein levels was significantly increased by treatment with ATD (Fig. 8C, D). Injection with E2 had no effect on nNOS mRNA and protein expression in normoxic fish, but the hypoxia-induced increase in nNOS mRNA and protein levels was partially blocked by treatment with E2 (Fig. 8B-D).

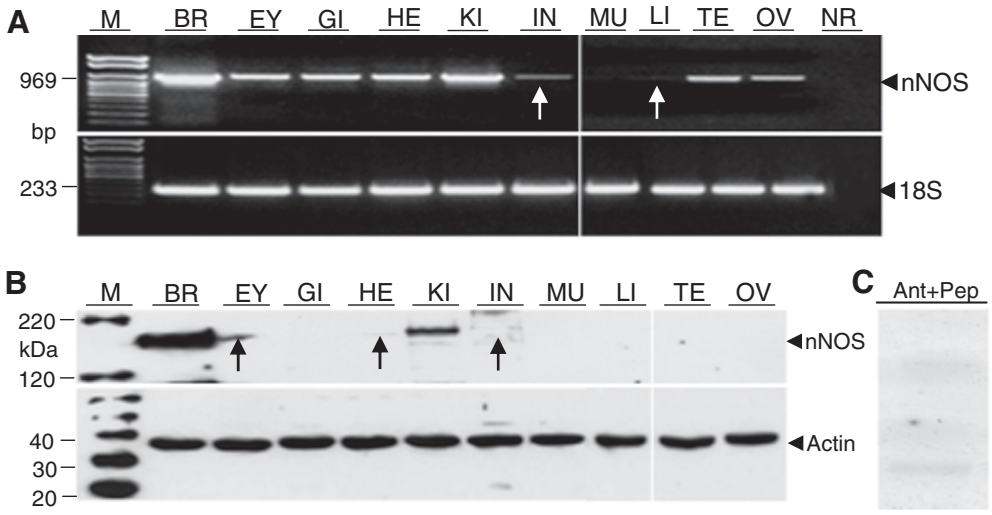

Fig. 3. Expression of nNOS mRNA and protein in croaker tissues. (A) RT-PCR analysis of nNOS mRNA in croaker tissues. Thirty-five PCR cycles were performed to detect nNOS mRNAs. (B) Immunoblot analysis of isolated nNOS protein using a polyclonal nNOS antibody. (C) Immunoreaction blocked by co-incubation of nNOS antibody with peptide antigen. BR, brain; EY, eye; GI, gill; HE, heart; IN, intestine; KI, kidney; LI, liver; TE, testis; OV, ovary; Ant, antibody; Pep, peptide. M, marker. The position of the DNA and protein markers (M) are indicated to the left of each image. bp, base pair; kDa, kilodalton. Arrows indicate faint expression of nNOS mRNA and protein. Note: due to gel size limitation, equal amounts of PCR products (Fig. 3A) and protein samples (Fig. 3B) from different tissues were run at room temperature under the same conditions on two separate gels. NR, negative reaction; kDa, kilodalton.

3.7. Co-localization of nNOS and aromatase proteins

To investigate the anatomical basis for possible interactions between nNOS and aromatase proteins in the croaker hypothalamus, we investigated their co-localization using double-immunofluorescence assay. Immunohistochemical results showed that nNOS and aromatase proteins were co-expressed in the croaker preoptic-anterior hypothalamus as shown in the merged images (Fig. 9A).

4. Discussion

The results of this study indicate the Atlantic croaker nNOS protein has high sequence similarity with other vertebrate nNOSs and show for the first time that chronic hypoxia exposure causes upregulation of nNOS expression the hypothalamic region of the fish brain, an oxygen-sensitive organ. Moreover, several lines of evidence demonstrate that the increase in nNOS expression after hypoxia exposure causes a marked rise in $O_2^{\bullet -}$

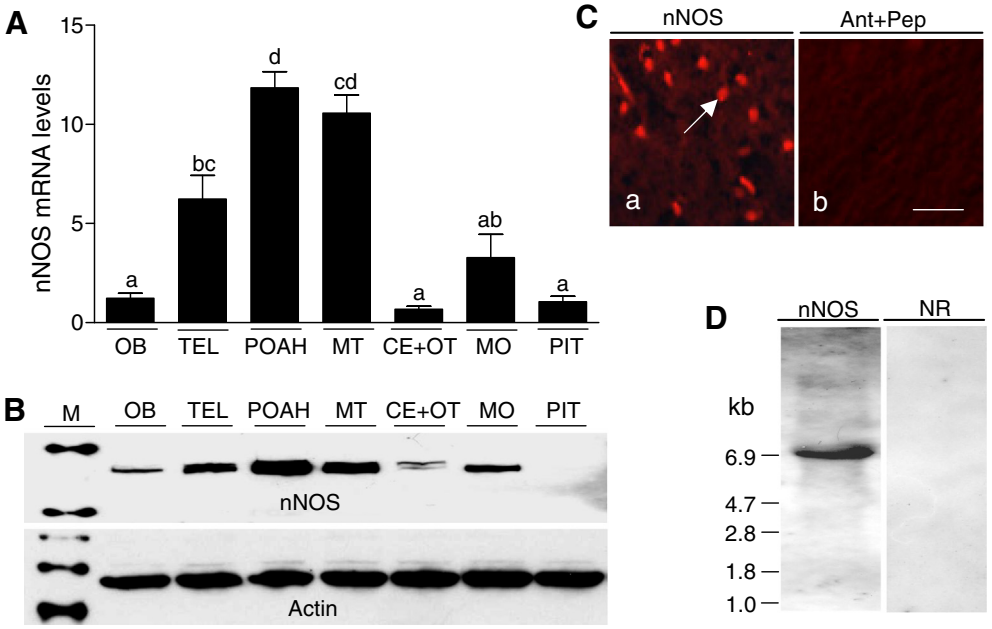

Fig. 4. Expression of nNOS mRNA and protein in croaker brain and pituitary. (A) Quantitative real-time PCR analysis of nNOS mRNA in the discrete brain areas and pituitary. Total RNA (200 ng) from each brain area and pituitary were used for 40 cycles of real-time PCR with nNOS qRT-PCR primers, and actin primers. Significant differences identified with a multiple range test, Fisher's PLSD, are indicated with different letters ($P < 0.05$). (B) Immunoblot analysis of isolated nNOS protein in the discrete brain areas and pituitary using a polyclonal nNOS antibody. (C) Immunohistochemical nNOS expression in croaker hypothalamus. Brain sections incubated with the primary and secondary antibodies showing the presence of immunoreactive signals (C-a, arrow indicates neuron in the hypothalamic area), and immunoreaction blocked by co-incubation of nNOS antibody with peptide antigen showing the absence of signal (C-b). Scale bar = 20 μm. (C) Northern blot analysis of nNOS transcript (transcript size ~7.0 kb) in croaker hypothalamus. OB, olfactory bulb; TEL, telencephalon; POAH, preoptic-anterior hypothalamic area; MT, midbrain tegmentum; CE + OT; cerebellum plus optic tectum; MO, medulla oblongata. M, protein marker. Ant, antibody; Pep, peptide; NR, negative reaction.

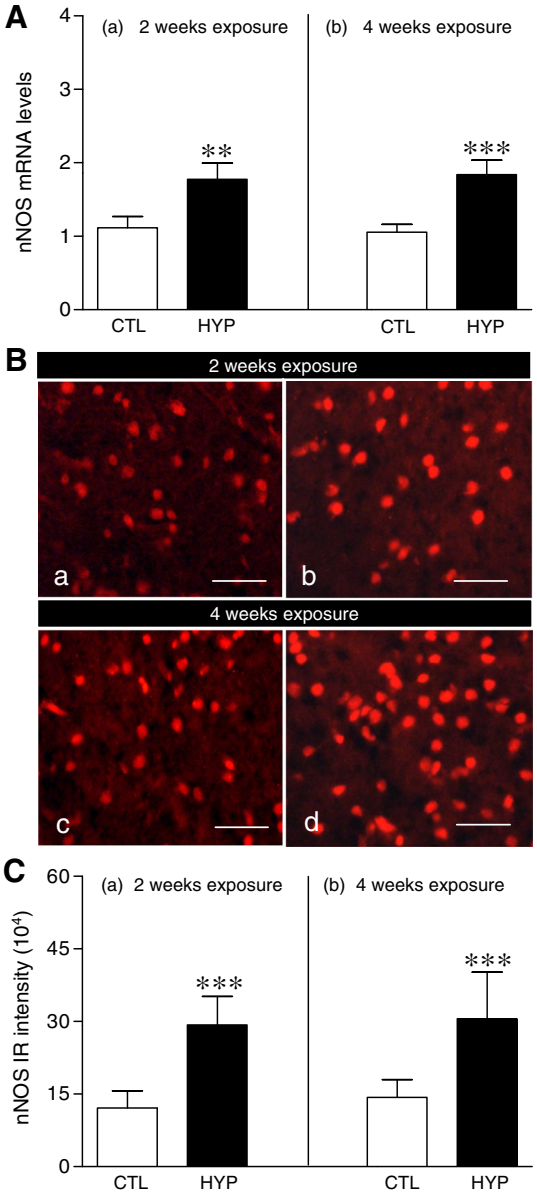

Fig. 5. Effects of two and four weeks hypoxia exposure (dissolved oxygen, DO: 1.7 mg/L) on mRNA levels and neuronal expression of nNOS in croaker hypothalamus determined by real-time qRT-PCR (A) and immunohistochemistry (B, C), respectively. Note: here and in subsequent figures exposure duration only refers to period fish were exposed to target DO, fish were previously exposed to declining DO for additional 2-day adjustment period. Each bar represents mean ± SE (N = 12, results from both sexes were combined because they were not significantly different) for mRNA levels (A). (B) Representative immunohistochemistry micrographs of nNOS neuron in hypothalamic sections (a, c: control; b, d: hypoxia exposure). Scale bar = 20 μm. (C) Immunoreactive (IR) staining intensity of the fluorescent-labeled nNOS neuron. Each value represents the mean ± SE (N = 30–35 neurons). Asterisk indicates significant difference (Student's *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$) between normoxic control (CTL) and hypoxia (HYP).

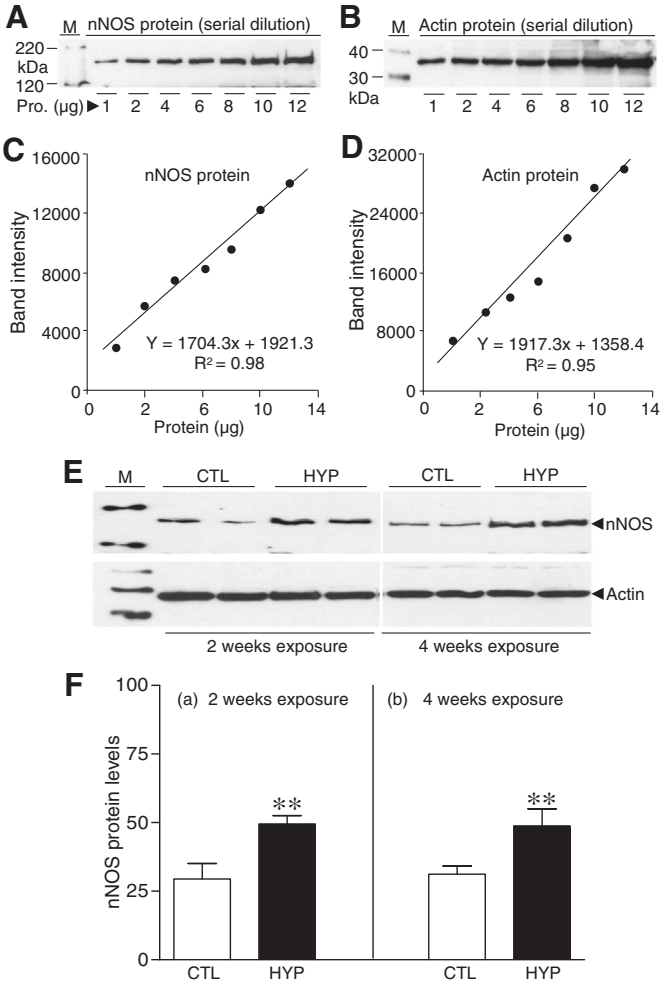

Fig. 6. Serial dilution of nNOS and actin protein expressions determined by Western blot analysis (A, B) and their immunoreactive intensities estimated by ImageJ software and from the regression lines (C, D). (E, F) Effects of two and four weeks hypoxia exposure (dissolved oxygen, DO: 1.7 mg/L) on nNOS protein expression and relative protein levels in croaker hypothalamus. Each bar represents mean ± SE (N = 10, results from both sexes were combined because they were not significantly different). Asterisk indicates significant difference (Student's *t*-test, $^{**}P < 0.01$) between normoxic control (CTL) and hypoxia (HYP). M, protein marker; kDa, kilodalton.

production in hypothalamic tissue. Importantly, the present study also provides the first clear evidence that an antioxidant and E2 have important protective roles in preventing the upregulation of nNOS expression and $O_2^{\bullet -}$ generation in the hypothalamus during hypoxic stress. Their mechanisms of actions were not investigated in this study so it remains unclear whether these protective functions involve intermediaries. The severe disruption of neuroendocrine and other hypothalamic functions in vertebrates after hypoxia exposure is associated with the generation of $O_2^{\bullet -}$ and other ROS (Tan et al., 1998). The present findings suggest that a multifaceted protective mechanism involving antioxidants and estrogens, regulates nNOS activity and prevents ROS generation under normoxic conditions. However, this mechanism is compromised under hypoxic conditions and exogenous antioxidant or estrogen administration is required to prevent ROS generation and ameliorate the effects of hypoxia on neuroendocrine functions (Fig. 9B). The roles of antioxidant and overall estrogen status in modulating the deleterious effects of hypoxia exposure and ROS generation on vertebrate brain functions and how they are altered under hypoxic conditions warrant further investigation.

The results from several experiments using various inhibitor, antioxidant and estrogen treatments support the involvement of nNOS in ROS generation and its regulation by antioxidants and estrogen. The finding that NOS-inhibitor (NAME) treatment completely blocked the hypoxia-induced upregulation of $O_2^{\bullet -}$ production and nNOS expression in the hypothalamic tissues suggests that the hypoxia-induced $O_2^{\bullet -}$ generation is mediated through the enzymatic activity of nNOS. The observation that NEM increased $O_2^{\bullet -}$ production and nNOS expression in normoxic control fish similar to that seen in hypoxia-exposed fish is consistent with a role of nNOS in ROS generation and indicates a potential mechanism of hypoxia-induced impairment of hypothalamic functions through modification of the sulfhydryl (SH) groups in the reactive centers of enzymes. The results showing that treatment with an antioxidant

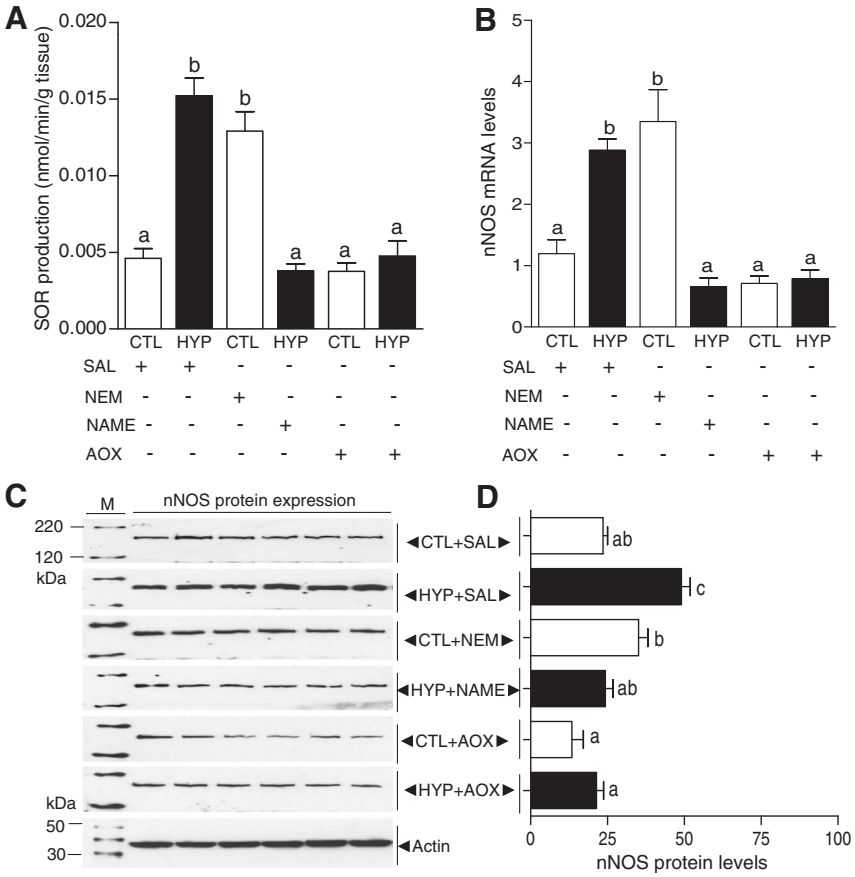

Fig. 7. Interactive effects of hypoxia and pharmacological agents that modulate generation of reactive oxygen species (ROS) and nNOS expression in croaker hypothalamus. Effects of hypoxia exposure (DO: 1.7 mg/L for 4 weeks) and pharmacological treatments with *N*-ethylmaleimide (NEM, a chemical which covalently modifies sulfhydryl groups); *N*ω-nitro-L-arginine methyl ester (NAME, an inhibitor of NOS) and vitamin E (an antioxidant, AOX) on superoxide radical (SOR, $O_2^{\bullet -}$, a ROS) production (A), nNOS mRNA levels (B), protein expression (C). Representative Western blot shown for samples from individual fish (note: a single representative Western blot actin loading control is shown), and relative protein levels (D) in croaker hypothalamus. Each bar represents mean ± SE (N = 9–12, results from both sexes were combined because they were not significantly different). Significant differences as compared to control (CTL) identified with a multiple range test, Fisher's PLSD, are indicated with different letters ($P < 0.05$). SAL, saline; HYP, hypoxia. HYP, hypoxia; SAL, saline; kDa, kilodalton.

prevents hypoxia-induced increases in nNOS and ROS production further supports this proposed mechanism because antioxidants have been shown to exert a protective effect on ROS-induced protein damage through SH modifications (Rachmilewitz et al., 1995). In addition, the demonstration that repeated injections with an aromatase-inhibitor increased hypothalamic $O_2^{\bullet -}$ production and nNOS expression in normoxic control fish suggests that maintenance of the E2 status is essential for preventing upregulation of ROS and nNOS. This is supported by the finding that E2 treatment significantly reduced $O_2^{\bullet -}$ production and nNOS expression in hypoxia-exposed fish. Previous studies have shown that hypothalamic aromatase activity is significantly decreased in croaker after chronic hypoxia exposure (Thomas and Rahman, 2012). Taken together, these results suggest that the increase in nNOS and ROS production after hypoxia exposure is due, at least partially, to a decline in hypothalamic aromatase activity and/or circulating E2 levels. Our results demonstrating co-localization of the nNOS protein with aromatase protein in the croaker hypothalami provides the anatomical basis for direct interactions between these two neuroenzymes. Collectively, our results suggest that hypoxia causes upregulation of nNOS expression which can be prevented by increasing the antioxidant and E2 status, resulting in down-regulation of $O_2^{\bullet -}$ production during hypoxia exposure. However, the mechanism of E2's action and whether it acting through an estrogen receptor-mediated mechanism, as well as details of the protective antioxidant mechanism in the hypothalamus, remain unclear.

The croaker nNOS deduced amino acid sequence possesses the conserved functional domains and characteristic features of other nNOS proteins which suggest it has similar functions to those of other vertebrate nNOSs. The finding that croaker nNOS mRNA is highly expressed in the brain but has relatively low expression in other tissues is consistent with previous results in other teleost fishes (Zhou et al., 2009; Yao et al., 2014). An anticipated finding in this study is that, like human nNOS protein expression (Park et al., 2000), croaker nNOS protein is largely restricted to the brain and to a lesser extent to the kidney. nNOS also showed weak expression in the eye, heart and intestine which suggests that NO and $O_2^{\bullet -}$ are may be synthesized by the nNOS in a variety of fish tissues (Andrew and Mayer, 1999).

It is noteworthy that croaker nNOS is highly expressed in the entire hypothalamic region where various neuropeptides, neurotransmitter and neuroenzymes controlling reproductive neuroendocrine functions in teleost fish are largely concentrated (Diotel et al., 2011; Gopurappilly et al., 2013). Low expression of nNOS mRNA and protein was also observed in the croaker olfactory bulb, optic tectum and medulla oblongata. These differential expression patterns of croaker nNOS mRNA and protein in different brain areas are consistent with the patterns of enzymatic activity, protein and immunoreactive expression of nNOS in discrete brain areas of goldfish and trout (Bruning et al., 1995; Virgili et al., 2001). In human brain, lowest nNOS mRNA expression was detected in the olfactory bulb and telencephalon, and highest expression was found in the cerebellum and putamem (Bredt et al., 1991; Park et al., 2000). In contrast, the highest NOS activity was measured in the striatum, while low activity was found in the cerebellum in the marmoset brain (Gerlach et al., 1995). On the other hand, highest nNOS activity was observed in the cerebellum and lowest activity in the spinal cord in rat and mouse brains (Barjavel and Bhargava, 1995). Collectively, these results suggest there is considerable

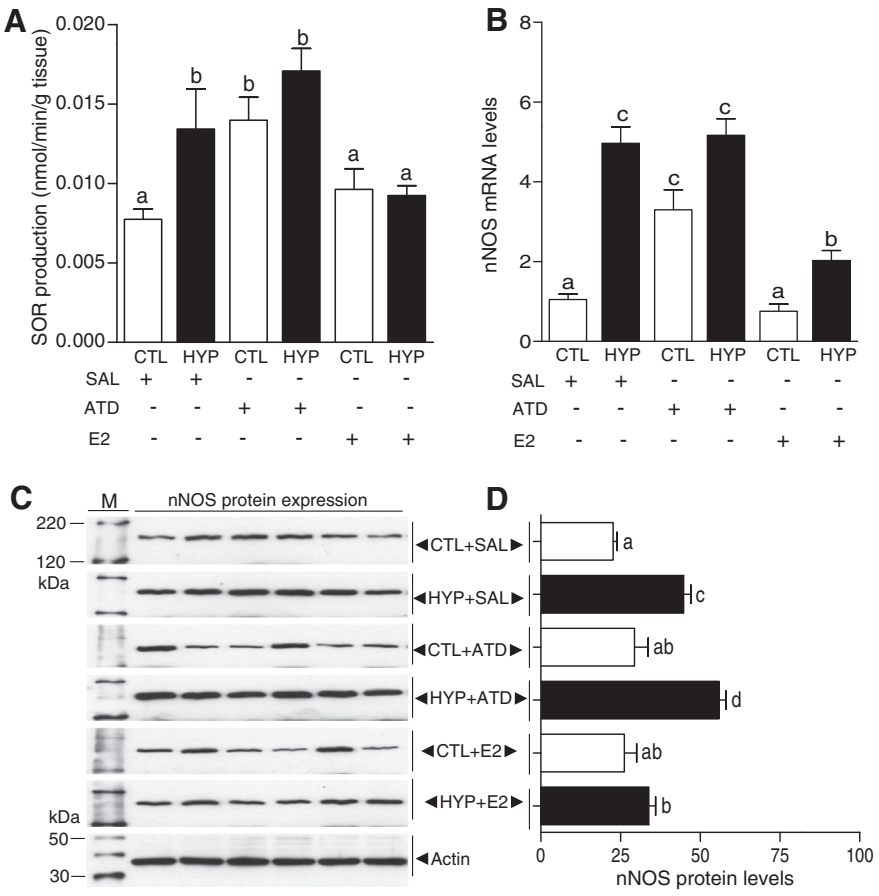

Fig. 8. Interactive effects of hypoxia and pharmacological agents that modulate generation of reactive oxygen species (ROS) and nNOS expression in croaker hypothalamus. Effects of hypoxia exposure (DO: 1.7 mg/L for 4 weeks) and pharmacological treatments with 1,4,6-androstatrien-3,17-dione (ATD, an aromatase inhibitor); estradiol-17β (E2, an aromatized C18 steroid) on superoxide radical (SOR, $O_2^{\bullet -}$, a ROS) production (A), nNOS mRNA levels (B), protein expression (C). Representative Western blot shown for samples from individual fish (note: a single representative Western blot actin loading control is shown), and relative protein levels (D) in croaker hypothalamus. Each bar represents mean ± SE (N = 9–12, results from both sexes were combined because they were not significantly different). Significant differences as compared to control (CTL) identified with a multiple range test, Fisher's PLSD, are indicated with different letters ($P < 0.05$). HYP, hypoxia; SAL, saline; kDa, kilodalton.

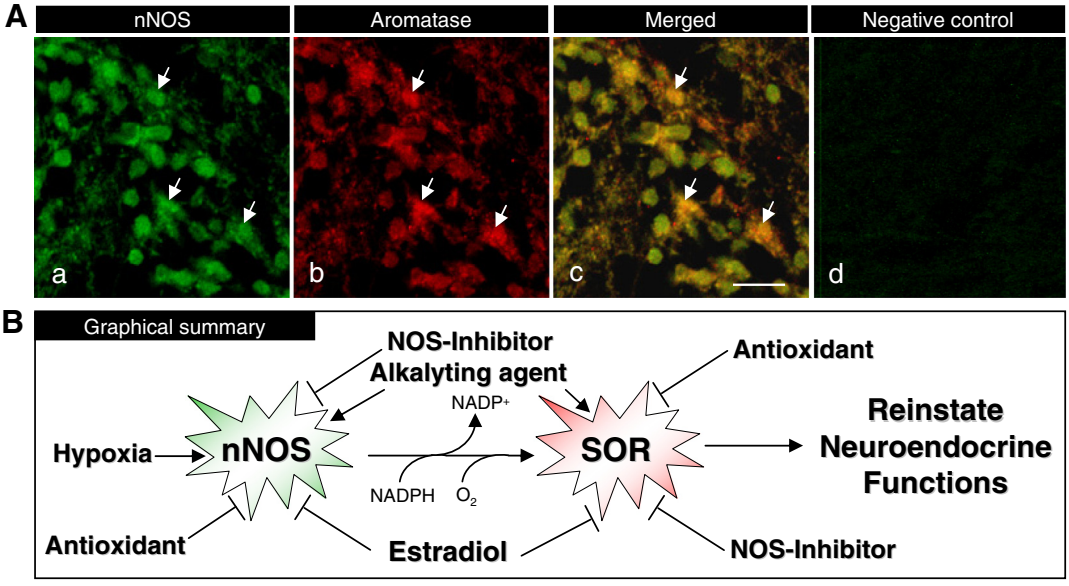

Fig. 9. Immunohistochemical co-localization of nNOS and aromatase proteins in croaker hypothalamus determined by double-immunofluorescence staining (A). Arrows indicate neuronal expression of nNOS, aromatase and their co-expression (merged). Scale bar = 10 μm. (B) Graphical summary of hypoxia-induced upregulation of superoxide radical (SOR, $O_2^{\bullet -}$, a ROS) through activation of nNOS in croaker hypothalamus. The antioxidant (Vitamin E), nitric oxide synthase (NOS)-inhibitor and estradiol-17β (E2) block hypoxia-induced upregulation of nNOS expression and SOR production and reinstate neuroendocrine functions. NADPH, nicotinamide adenine dinucleotide phosphate.

species variation in nNOS expression in different brain regions, although its significance is unknown.

Molecular analysis has shown that alternative splicing of nNOS can occur, a process of gene expression that results in a single gene coding for multiple proteins (Black, 2003), that allows the creation of nNOS proteins differing in both enzymatic characteristics and structural features (Brenman et al., 1996; Eliasson et al., 1997). In mammals, several sizes and/or alternatively spliced forms of nNOS mRNA transcripts have been identified by Northern blot analyses (Brenman et al., 1996; Eliasson et al., 1997). For example, Brenman et al. (1996) showed that a probe corresponding to the full coding region of nNOS hybridized to a 9.5-kb band in adult rat brain and embryonic tissues, but hybridized to a 2.5-kb band in adult testis. In mice, the nNOS mRNA transcript was identified at ~9.5 kb in the brain (Huang et al., 1993) and at ~10 kb in the adductor muscle (Pagel et al., 2011). In contrast a single nNOS mRNA transcript ~10 kb was found in human brain, placenta, liver and skeleton muscle (Nakane et al., 1993; Park et al., 1996). Similarly in a teleost, multiple nNOS mRNA transcripts were identified in Atlantic salmon brains, of approximately 7.0 kb, 9.5 kb and a transcript slightly larger than 9.5 kb in the optic tectum (Oyan et al., 2000). However, the finding that a single hybridization signal was detected in croaker hypothalamic tissues by Northern blot analyses suggests that only a single ~7 kb nNOS transcript is present in this species.

There is growing evidence that hypoxia exposure increases nNOS expression in variety of vertebrate species (Yamamotoa et al., 2003; McNeill and Perry, 2006; Mishra et al., 2006; Tsui et al., 2011). An important finding in the present study is that short- and long-term exposure (2 and 4 weeks) to hypoxia (DO: 1.7 mg/L) causes marked increases in nNOS mRNA, protein and neuronal expression in the croaker hypothalamus. Similarly, short-term hypoxia exposure for four days has been shown to increase nNOS mRNA levels in the brain and posterior cardinal vein in rainbow trout, which is accompanied by increases in plasma NOx, a NO metabolite levels (McNeill and Perry, 2006). In addition, short-term chronic hypoxia exposure (DO: 0.39 mg/L for 2 h) caused a marked increase the immunoreactive neuronal expression of NADPH-diaphorase (NADPHd, a marker for nNOS activity) in the brain and spinal cord of epaulet shark, an elasmobranch (Gillian et al., 1999). Similarly, short-term hypoxia leads to increase NADPHd immunoreactive expression in the nodose ganglion neurons in the rat brain (Yamamotoa et al., 2003) and nNOS mRNA and protein levels in the brains of piglets and mice (Mishra et al., 2006; Tsui et al., 2011). Although Li et al. (2004) demonstrated that short-term hypoxia (1–3 days) increased NOS activity, NOx production and nitrotyrosine, a marker of NO-derived species such as $ONOO^-$, immunoreactive expression in the rat cortex; no significant effect on nNOS protein expression was observed. On the other hand, long-term cyclic hypoxia (exposure cycle: every 2 min diurnally for 8 h/day, 6 days/week for 5 weeks) decreased nNOS immunoreactive expression and mRNA levels in the paraventricular hypothalamic tissues in rat (Huang et al., 2007). Collectively, these results suggest that the pattern of changes in hypoxia-induced nNOS activity and expression differs among teleost and tetrapod tissues under different hypoxia exposure paradigms.

Hypoxia-induced oxidative stress can generate cellular reactive free radicals such as $O_2^{\bullet -}$ and $ONOO^-$ via transcriptional and/or post-translation mechanism(s) to alter expression of NOS (Förstermann, 2006; Pascali et al., 2014). The finding that NEM, an alkylating agent which covalently modifies SH groups and inhibits aromatase activity (Snyder et al., 1996), mimics the effects of hypoxia on $O_2^{\bullet -}$ production and nNOS mRNA and protein expression in croaker hypothalamus is consistent with such a mechanism. NEM also alkylates the important antioxidant, glutathione (GSH) activity (Schulz et al., 2000). Heales et al. (1996) demonstrated that the depletion of GSH is accompanied by increased nNOS activity in the rat brain. In addition, treatment with alkylating agent such as iodoacetamide increases NOS activity and NOx levels in rat colonic segments under normoxic conditions (Rachmilewitz et al., 1995). On the contrary, treatment with GSH decreases NOS activity in rat hepatocytes *in vitro* under normoxic conditions (Harbrecht et al., 1997). Taken together, these results suggest that restricting the catalytic activity and post-translational regulation of NOS is dependent on the presence of sufficient endogenous SH residues and that declines in cellular GSH content may trigger ROS and RNS generation and NOS activation during hypoxia exposure (Schulz et al., 2000). In support of this proposed mechanism there is considerable evidence that hypoxia decreases total SH residues and GSH contents, leading to increases in ROS and RNS levels in the brain and hepatic tissues in vertebrates (Wideman and Domanska-Janik, 1974; Iyer et al., 1985; Farooqui, 2012).

The present results showing that the hypoxia-induced increases in hypothalamic $O_2^{\bullet -}$ production and nNOS expression are blocked by administration of Vit E are consistent with our previous results showing that the hypoxia-induced increases in $O_2^{\bullet -}$ production and eNOS expression in croaker livers are also attenuated by Vit E (Rahman and Thomas, 2011, 2012). In addition to hypoxia-induced cellular reactive free radicals, cellular cytokines (CTK) as well as lipid peroxidation (LPD) also promote oxidative modifications and play an important role in neuronal injury as well as elevation of NOS activity (Gadek-Michalska et al., 2013). It has been shown that during hypoxic stress, CTK such as interleukin-1β (IL-1β), tumor necrosis factor, and interferon-γ, are synchronously increased in tetrapod tissues and that elevated CTK transiently enhances the production of $O_2^{\bullet -}$ via activation of NOS (Haddad and Har, 2005). Numerous tetrapod studies have also shown that hypoxia markedly decreases cellular levels of antioxidants such as Vit C and Vit E which leads to increased cellular oxidative stress and neuronal cell death (Chow, 1991; Traber and Stevens, 2011; Magalhães et al., 2005). Interestingly, an *in vivo* study in rats showed that hypoxia caused significant increases in neuronal LPD, NOx, and protein carbonyls (PC, an indirect measure of ROS) levels with a concomitant decrease in total antioxidant capacity compared with controls (Tan et al., 1998). Moreover, the hypoxia-induced increases in LPD, NOx and protein oxidation were completely blocked by administration of various antioxidants such as ascorbic acid and trolox (Tan et al., 1998). These results are consistent with the results of our previous *in vivo* studies in croaker showing that hypoxia markedly increases hepatic IL-1β mRNA levels, $O_2^{\bullet -}$ production and PC contents, and plasma NOx levels which are attenuated by treatment with Vit E (Rahman and Thomas, 2011, 2012, unpub. obs.). Importantly, antioxidants are prototypical scavengers of ROS, and attenuate CTK-induced NOS activation, and reduce LPD activity (Haddad and Har, 2005). Collectively, our findings together with those in tetrapods suggest that during hypoxia stress, Vit E prevents the upregulation of CTK and LPD activation, leading to decreases ROS and RNS generation in the vertebrate brain.

The most significant finding of this study is that inhibition of aromatase activity by ATD causes marked increases in hypothalamic $O_2^{\bullet -}$ production and nNOS mRNA and protein expression, similar to those observed in hypoxia-exposed fish, which was reversed by E2 treatment. *In vivo* studies in tetrapods have shown that E2 decreases nNOS neuronal expression in rat hypothalamic paraventricular (PV) nuclei and anterior pituitary (Qian et al., 1999; Gingerich and Krukoff, 2005) and nNOS protein expression in nerves and ganglia of the vagina and clitoris of rabbits under normoxic conditions (Yoon et al., 2001). In addition, *in vitro* studies in tetrapods have shown that E2 decreases nNOS immunoreactive expression in rat hypothalamic slice cultures under normoxic conditions (Gingerich and Krukoff, 2005). On the contrary, *in vivo* studies have shown that E2 increases NOS activity in male rat adipocytes (Jaubert et al., 2007) and nNOS mRNA levels in the ventral medial nucleus and the hippocampus in the brains of ovariectomized rats (Ceccatelli et al., 1996; Grohe et al., 2004). Other studies have reported no changes in nNOS mRNA and protein levels and neuronal nNOS expression in the magnocellular, supraoptic and PV nuclei after E2 treatments (Ceccatelli et al., 1996; Wang and Morris, 1999). The significance of these divergent findings is unclear, but they may be due to the fact that the effects of E2 on nNOS regulation in the vertebrate brain appear to be

regionally specific. Studies *in vivo* have shown that E2 suppresses mitochondrial oxidative stress and NO production in rat brain and uterine tissues (Yallampalli and Dong, 2000; Razmara et al., 2007). Furthermore, recent *in vitro* studies have shown that E2 inhibits H_2O_2, $O_2^{\bullet-}$ and $ONOO^-$ production in the rat brain mitochondria, PC12 cells, and human umbilical vein endothelial cells, respectively (Razmara et al., 2007; Borrás et al., 2010; Chakrabarti et al., 2012). E2 regulates neuroenzyme activity and ROS generation through an estrogen receptor mechanism (McEwen and Alves, 1999; Grohe et al., 2004). It has been shown that E2 acts via a nuclear estrogen receptor (ER)-dependent genomic pathway to influence nNOS regulation in mammals (Molero et al., 2002; Gingerich and Krukoff, 2005). Gingerich and Krukoff (2005) demonstrated that the ER antagonist ICI 182,780 blocked the E2-induced effects on the number of nNOS-immunoreactive positive neurons in the rat hypothalamic PV nuclei. These authors also suggested that ERβ mediates the E2-induced changes in NOS expression in the rat PV nuclei. Indeed, multiple ERβ subtypes (ER-βa and ER-βb) in addition to ERα are expressed in teleosts (Diotel et al., 2011) including croaker brain (Hawkins et al., 2005). In addition, recent evidence indicates G protein coupled receptor 30 (GPR30) is an intermediary in estrogen regulation of NO production in cardiovascular tissues (Meyer et al., 2011; Holm and Nilsson, 2013). Therefore, further studies using ER and GPR30 antagonists and agonists need to be conducted in order to determine the potential molecular mechanism of E2 action on nNOS and ROS regulation in the vertebrate brain during hypoxic stress.

In conclusion, the data presented in this study demonstrate for the first time in an aquatic vertebrate that hypoxia increases $O_2^{\bullet-}$ generation and nNOS mRNA and protein expression in the hypothalamus, an important integrative center in the fish brain. The results also show that during hypoxic stress, administration of Vit E or E2 leads to reduced $O_2^{\bullet-}$ generation and nNOS expression in the fish hypothalamus. This is the first evidence for an involvement of Vit E and E2 in reversing hypoxia-induced upregulation of nNOS and ROS in the teleost brain.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2015.03.013.

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